Reference: *Biol. Bull.* **167**: 445–459. (October, 1984)

CHEMOAUTOTROPHIC SYMBIONTS IN THE BIVALVE *LUCINA FLORIDANA* FROM SEAGRASS BEDS

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ABSTRACT

Enzymatic and histological evidence suggest that the eulamellibranch bivalve *Lucina floridana* possesses bacterial endosymbionts capable of a chemoautotrophic metabolism. Dense populations of *L. floridana* (83 ± 11 per m^2; 95% CI, n = 33) are found closely associated with the O_2-releasing root systems of seagrasses in sulfide-rich sediments; the sandy sediments of both *Thalassia* and *Ruppia* beds contain 1.67 ± 0.31 m*M* (95% CI, n = 13) and 2.49 ± 0.55 m*M* (95% CI, n = 13) sulfide, respectively. Both transmission electron microscopy of gill tissue and scanning electron microscopy of freeze-fractured gills reveal numerous rod-shaped procaryotic inclusions in vacuoles of large, eucaryotic cells ("bacteriocytes") located deeply within demibranch cross sections; no such inclusions are seen in the ciliated gill epithelium which is rich in mitochondria. Activities of ribulose 1,5-bisphosphate carboxylase (RuBPCase), phosphoribulokinase, APS reductase, ATP sulfurylase, and nitrite reductase have been measured and partially characterized in homogenates of fresh gill tissue. Light microscopy reveals numerous aggregations of pigmented granules localized to the interior of the gill in association with the bacteriocytes. Histochemical staining demonstrates the presence of iron in these granules, consistent with the idea that their composition, in part, may be respiratory pigment and/or iron-containing cytochromes. Energy dispersive X-ray analysis reveals sulfur as a dominant inorganic element in the gill tissue. Based on abundance data of *L. floridana* and *in vitro* levels of RuBPCase (half-maximal velocity) this bivalve could potentially contribute 336 ± 96 g C/m^2/year (95% CI) to the gross carbon fixation of seagrass beds.

INTRODUCTION

Seagrass beds are now recognized as one of the most productive of marine communities, with primary production values of 500–1000 g C/m^2/year; high production areas may reach over twice this figure (Fenchel, 1977; Zieman and Wetzel, 1980). Since a large portion of the plant material produced is eventually deposited on the bottom as detritus (Kikuchi and Peres, 1977), the abundance of organic material in the sediment is often greater than the oxygen available for its degradation. Under these anaerobic conditions, decomposition is accomplished by the activity of certain bacteria which can utilize inorganic compounds other than O_2 as electron acceptors; $SO_4^=$, NO_3^-, CO_2, and H_2O may be reduced to H_2S, NH_3, CH_4, and H_2 (Fenchel and Riedl, 1970). Hydrogen sulfide is often the major inorganic constituent in the sediments due to the high availability of sulfates in sea water and the large populations of sulfate-reducing bacteria, such as *Desulfovibrio* sp. (Jorgensen and Fenchel, 1974).

Normally, metazoans are poorly represented in this sulfide biome, the predominant

Received 17 May 1984; accepted 24 July 1984.

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members being facultative/obligate anaerobes from the platyhelminthes and the aschelminthes (Fenchel and Riedl, 1970). However, oxygen is known to diffuse from the roots of marine angiosperms (Teal and Kanwisher, 1966; Armstrong, 1970) forming aerobic zones in the immediate vicinity of the root systems (oxidized rhizospheres), and it is among the roots of *Thalassia* and *Ruppia,* two seagrasses common to high-salinity, coastal waters of the Gulf of Mexico (Edwards, 1976), that the eulamellibranch bivalve *Lucina floridana* is known to occur (Britton, 1970). This habitat would potentially provide this bivalve with simultaneous access to both oxygen and hydrogen sulfide. Such an interface between these two molecular species is very similar to that documented for the effluent waters of the hydrothermal vents along the Eastern Pacific Rise (*e.g.,* see Edmond *et al.,* 1982). Consequently, we chose to study selected enzyme systems and the gill ultrastructure of the shallow-living bivalve *L. floridana* to determine, first, if the potential for chemoautotrophic metabolism was present in this species, as was described for certain hydrothermal vent organisms (Cavanaugh *et al.,* 1981; Felbeck, 1981; Felbeck *et al.,* 1981; Felbeck and Somero, 1982; Cavanaugh, 1983). The vestimentiferan tube worm *Riftia pachyptila,* the clam *Calyptogena magnifica,* and the unidentified vent mussel possess symbiotic sulfur-oxidizing bacteria capable of a chemoautotrophic metabolism. More recently, species of non-hydrothermal vent bivalves have also been identified as having sulfur-based metabolism (Cavanaugh, 1983; Felbeck, 1983; Berg and Alatalo, 1984). Second, clarifying the spatial arrangement and position of the endosymbionts relative to other tissue structures (*e.g.,* the distinctive pigment granules of lucinid gills) could indicate the degree of access of molecular species like oxygen and sulfide to the bacteria. Finally, such a chemoautotrophic metabolism, if present in *L. floridana,* could represent a significant amount of gross carbon assimilation, which has as yet been unappreciated for seagrass ecosystems.

Materials and Methods

Specimen collection and population densities

Specimens of *L. floridana* were collected from the turtle grass (*Thalassia testudinum*) and widgeon grass (*Ruppia maritima*) beds along the western shore of St. Joseph's Bay, Florida, in 0.25–1.0 m of water, and from the widgeon grass beds along the mainland shore of Alligator Harbor, Florida at a depth of 1–2 m. Specimens were maintained in their original substrate (sulfide rich) in 20 gallon aquaria at 32 ppt salinity. All experimental animals were used within three weeks of collection, although they routinely survived several months under the above conditions in the laboratory. Population densities at the St. Joseph's Bay site were measured with a 0.1 m^2 quadrat thrown from three separate transects at intervals of 30, 45, 60, 75, and 100 meters from shore. All specimens were collected from a sediment depth of 0–20 cm; no specimens were found below 20 cm.

Sulfide measurements

Sulfide concentrations were determined in sediments at depths of 5, 10, 15, and 20 cm in both *Thalassia* and *Ruppia* beds, using sections sliced from core samples. Sediment samples were diluted 1:1 (w/v) with a sulfide antioxidant buffer consisting of 80 g NaOH, 320 g sodium salicylate, and 72 g ascorbic acid in 1000 ml distilled water (modified after Baumann, 1974). All samples were voided of air bubbles and kept on ice in airtight bags. Sulfide measurements were made within 36 hours with an Iotrode AB 120 sulfide electrode and an Orion 407A specific ion meter. The amount of interstitial water was analyzed by drying a fresh sediment sample of known

weight at 90°C for 36 hours. The difference between wet and dry weights was then used as a measure of interstitial water.

Enzymatic activity determinations

All enzymatic assays were performed using fresh gill tissue which was homogenized in three volumes of the appropriate, ice-cold medium with a Wheaton ground glass homogenizer. The crude homogenates were centrifuged at 5000 × *g* for 15 minutes and the supernatants were saved and used for the assays without further purification.

Ribulose 1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] activity was measured using the protocol as described by Wishnick and Lane (1971). The homogenizing medium consisted of 10 m*M* tris-HCl buffer (pH 7.8), 0.1 m*M* EDTA, and 10 m*M* β-mercaptoethanol. The 0.5 ml reaction mixture contained 100 μmoles tris-HCl buffer (pH 7.6), 5 μmoles $MgCl_2$, 25 μmoles $NaHCO_3$, 0.03 μmoles EDTA, 3.0 μmoles glutathione, 1.0 μmole ribulose 1,5-bisphosphate, and 2 μCi of $NaH^{14}CO_3$ (specific activity, 50 μCi/μmole). The reaction mixture was incubated for 10 minutes at 20°C, stopped with 6 *N* HCl, and heated at 90°C for 60 minutes. Radioactivity incorporated into the acidified sample was measured by liquid scintillation counting.

The method of Racker (1975) was followed for the assay for phosphoribulokinase (ATP:D-ribulose-5-phosphate 1-phosphotransferase, EC 2.7.1.19). Gill tissue was homogenized in 1.0 *M* tris-HCl buffer (pH 7.8). The assay mixture contained 50 μmoles tris-HCl buffer (pH 7.8), 10 μmoles $MgCl_2$, 10 μmoles glutathione, 12 μmoles ATP, 75 μmoles $KHCO_3$, 0.15 μmoles NADPH, 5 μmoles ribose-5-phosphate, 5 units ribose-5-phosphate isomerase, 5 units glyceraldehyde-3-phosphate dehydrogenase, and 5 units phosphoglycerate kinase in a final volume of 1.0 ml. The oxidation of NADPH was followed at 340 nm.

The method described by Peck *et al.* (1965) was used for the assay of APS reductase [AMP, sulfite:(acceptor) oxidoreductase, EC 1.8.99.2]. The gill tissue was homogenized in 0.3 *M* tris-HCl buffer (pH 8.0) and 5 m*M* EDTA. The 3.0 ml reaction mixture contained 300 μmoles tris-HCl buffer (pH 8.0), 10 μmoles AMP, 4 μmoles sodium ferricyanide, and 10 μmoles sodium sulfite. The reduction of the ferricyanide ion was monitored by measuring the decrease in absorbance at 410 nm (λ max). The millimolar extinction coefficient of oxidized ferricyanide in distilled water was empirically determined to be 0.927 $millimoles^{-1}$ cm^{-1} at 410 nm. Background rates of the reaction mixture without AMP were subtracted to correct for sulfite oxidase activity.

ATP sulfurylase (ATP:sulfate adenyltransferase, EC 2.7.7.4) was assayed according to the method of Felbeck (1981). The homogenizing medium consisted of 0.1 *M* triethanolamine-HCl buffer (pH 7.3). The assay medium consisted of 0.1 *M* triethanolamine-HCl buffer (pH 7.3), 2.5 μmoles magnesium acetate, 25 μmoles glucose, 25 μmoles pyrophosphate, 0.05 μmoles $NADP^+$, 0.05 μmoles adenosine phosphosulfate, 2.5 units hexokinase, 1.25 units glucose-6-phosphate dehydrogenase, and 1 μmole P^1, P^5-di(adenosine-5′) pentaphosphate in a total volume of 2 mls. The reduction of $NADP^+$ was followed at 340 nm.

Rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.8.1) activity was assayed following the method of Smith and Lascelles (1966). Gill tissues were homogenized in 0.3 *M* tris-HCl buffer (pH 8.7). The 3 ml reaction mixture consisted of 300 μmoles tris-HCl buffer (pH 8.7), 150 μmoles NaS_2O_3, 200 μmoles half-neutralized sodium cyanide (NaCN:HCl, 2.5:1 molar ratio), 0.5 μmoles 2,6-dichlorophenol-indophenol (DCIP), and 25 mg phenazine methosulfate (PMS). The reduction of DCIP was followed at 600 nm.

Finally, nitrite reductase (ammonia:ferredoxin oxidoreductase, EC 1.7.7.1) activity was determined by the procedure described by Losada and Paneque (1971). The homogenizing medium consisted of 0.5 *M* tris-HCl buffer (pH 8.0). Each reaction flask contained, in a volume of 2 mls, 150 μmoles tris-HCl buffer (pH 8.0), 4 μmoles $NaNO_2$, 1.5 μmoles methyl viologen, and 44 μmoles sodium dithionite, added from a stock solution prepared in 0.29 *M* $NaHCO_3$. The reaction mixture was incubated for 30 minutes at 20°C, and the amount of substrate remaining in each reaction flask was determined colorimetrically (Losada and Paneque, 1971).

Protein analysis

For all gill homogenates the protein concentration in the supernatant was measured using the technique of Peterson (1977).

Scanning electron microscopy

Gill tissues were fixed with 5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4) to which sucrose was added to bring the final osmolality to 1200 mOsm/kg. Postfixation was carried in 0.1 *M* phosphate buffer (pH 7.4) containing 2% osmium tetroxide, and dehydration was accomplished by a graded acetone series. The dehydrated tissue was immersed in liquid nitrogen and fractured with a precooled razor blade. After critical point drying, the fractured tissues were mounted on a stub and sputter coated with gold-palladium.

Energy dispersive X-ray analysis

Tissues were fixed with 5% glutaraldehyde as above, but were not postfixed or dehydrated using organic solvents. Instead, they were allowed to air dry in a desiccator at room temperature for 48 hours, freeze-fractured as above, mounted, and coated by carbon vapor deposition. Tissues were not critical point dried.

Transmission electron microscopy

Materials were fixed in 0.3 *M* PIPES (pH 7.8) containing 4% paraformaldehyde and 5% glutaraldehyde. The osmolality was adjusted to 1200 mOsm/kg with filtered sea water. Tissues were postfixed with 2% osmium tetroxide in 0.3 *M* PIPES (pH 7.8) and dehydrated in a graded acetone series. Using the rapid infiltration method described by Millonig (1976), tissues were placed in a Spurr's low-viscosity resin/acetone mixture (1:1) on a rotator for 30 minutes and then transferred to 100% Spurr's and centrifuged at 1250 × *g* for 20 minutes. The centrifugation step was repeated once using fresh resin. Specimens were embedded in fresh Spurr's for 18 hours at 50°C, and sections were stained with 5% uranyl acetate and 0.1% lead citrate.

Histochemical staining

Gill tissue to be stained for the presence of iron was fixed with 5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4) and embedded in Spurr's low viscosity resin. Then 0.5 micron sections were prepared and stained for occult iron as described by Read (1962). Briefly, the procedure consisted of placing the sections into a 30% solution of H_2O_2 for two minutes, rinsing in distilled water, and staining the sections in a heated and acidified solution of 0.06 *M* potassium ferricyanide for fifteen minutes. The tissue was then counter-stained in 1% carminic acid after washing in distilled water. Finally, the sections were differentiated in a 4% potassium aluminum sulfate solution for five minutes and thoroughly rinsed in distilled water.

RESULTS

Hydrogen sulfide levels and population densities

Hydrogen sulfide occurs in the interstitial water of the sediments of both *Thalassia* and *Ruppia* beds at concentrations of 1.67 ± 0.31 m*M* (95% CI, n = 13) and 2.49 ± 0.55 m*M* (95% CI, n = 13), respectively. The compound appears to be uniformly distributed throughout these sandy sediments as a function of depth, since HS^- concentrations were not significantly different at depths of 5, 10, 15, or 20 cm (ANOVA, 95% confidence). Based on data collected from both seagrass beds (Table I), *Lucina floridana* exists in population densities of 83 ± 11 per m^2 (95% CI, n = 33). No statistical difference was shown between the densities found in *Thalassia* beds *versus* *Ruppia* beds (ANOVA, 99% confidence). However, live *Lucina* specimens were not observed in study areas entirely devoid of seagrasses, nor did the bivalves occur at sediment depths greater than 20 cm. This sediment depth corresponds to the maximum depth of root extension into the substratum at our grass bed sites.

Gill ultrastructure and histochemical staining

Both transmission electron microscopy of gill tissue and scanning electron microscopy of freeze-fractured gills reveal densely packed assemblages of rod-shaped procaryotic inclusions (Fig. 1A–C). The bacteria range from 4 to 6 microns in length (1–1.5 microns in diameter) and appear to be enclosed within vacuoles; the scanning electron micrograph seen in Figure 1A illustrates the high density of bacteria within the tissue as well as empty vacuolar spaces where bacteria have apparently fallen out during the freeze-fracturing process. In the higher magnification micrograph (Fig. 1B) bacteria residing within vacuoles are also visible just beneath the freeze-fracture plane. Figure 1C is a transmission electron micrograph of these inclusions showing their procaryotic-like structures (*i.e.*, no membrane-bound organelles and a distinct nuclear zone) and the vacuolar membrane that surrounds them.

The distribution of bacteria within the gill is not random, but rather, is restricted to a particular cell type. Figure 2A shows the ciliated gill epithelium to be a simple columnar epithelium composed of cells with large nuclei and an abundance of smaller inclusions, which at higher magnification are identifiable as mitochondria (Fig. 2A, inset). Bacteria have not been observed in this outer cellular layer. However, underlying the gill epithelium is a tissue composed of very large cells (up to 60 microns in diameter) which are rich in bacteria-containing vacuoles (Fig. 2B). In fact, the bacteria are easily the most abundant inclusion in the ground substance of the bacteriocyte. Autophagic lysosomes containing myelin-like figures and small vesicles are occasionally

TABLE I

Hydrogen sulfide concentrations in the interstitial water of sediments and population densities of L. floridana *in* Thalassia *and* Ruppia *seagrass beds*

Seagrass beds	Hydrogen sulfide (m*M*)	Population density of *L. floridana* (per m^2)
Thalassia	1.67 ± 0.31 (n = 13)	84 ± 12 (n = 21)
		83 ± 11 (average for collection site; n = 33)
Ruppia	2.49 ± 0.55 (n = 13)	74 ± 34 (n = 9)

An overall average for *Lucina* density is also presented which reflects the relative contribution from each seagrass type at the collection site. Values represent means plus or minus 95% confidence limits.

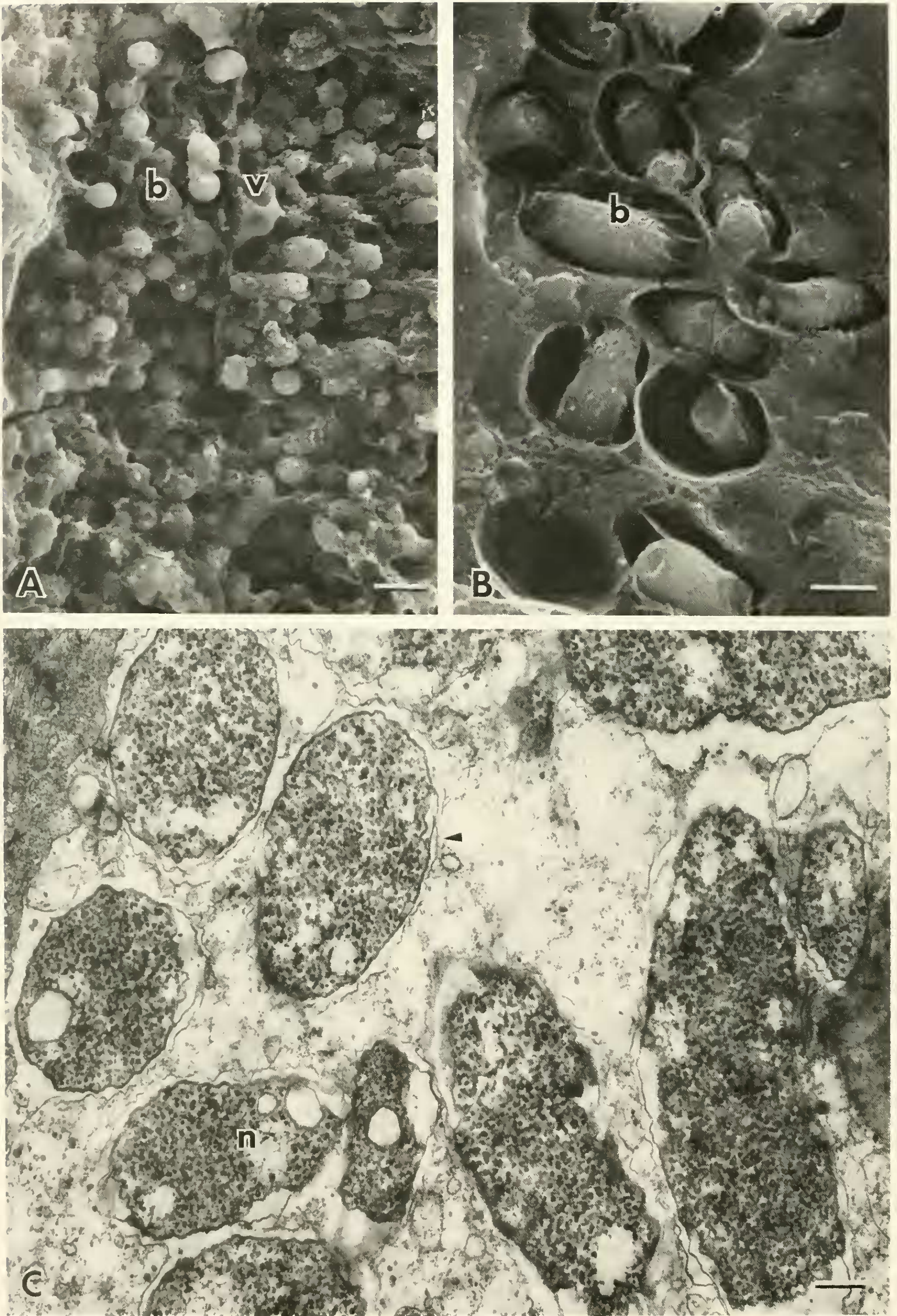

FIGURE 1. A. Scanning electron micrograph of freeze-fractured gill tissue, showing numerous bacteria (b) and empty vacuoles (v). Scale bar = 5 μm. A higher magnification is illustrated in B, where bacteria are also visible beneath the freeze-fracture plane. Scale bar = 2 μm. C. Transmission electron micrograph of the endosymbionts, which demonstrates their procaryotic structure, as noted by the nuclear region (n) and the lack of membrane-bound organelles. Arrow indicates vacuolar membranes surrounding a bacterium. Rarely is there more than one bacterium per vacuole. Scale bar = 0.5 μm.

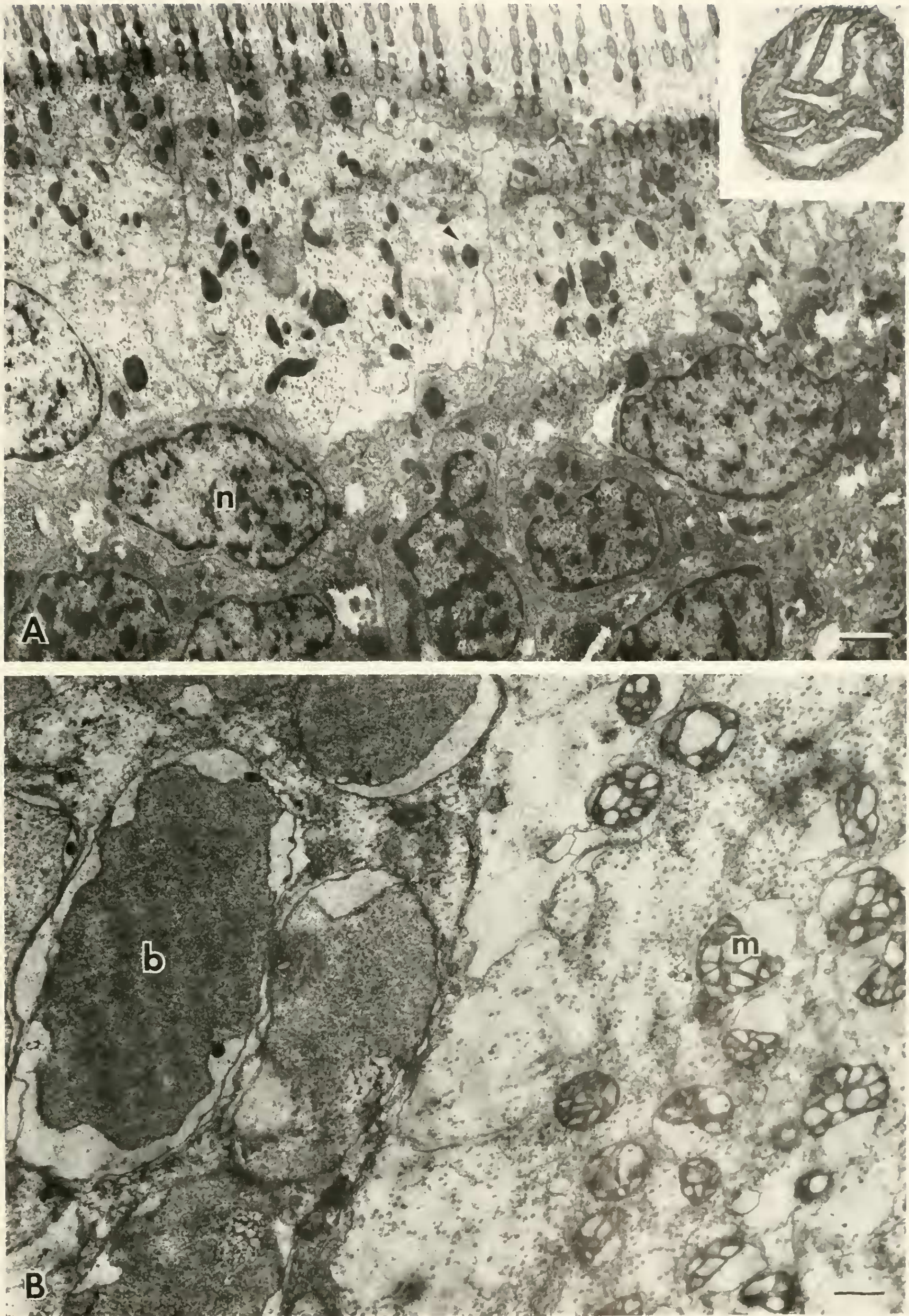

FIGURE 2. A. Transmission electron micrograph of a thin section through the gill epithelium, which is identifiable by its ciliated surface. Several large nuclei (n) are visible. Scale bar = 1 μm. The small inclusions (arrow) are mitochondria, as shown by the higher magnification in the inset. The lower micrograph, B, represents an interface between a bacterial-containing cell (bacteriocyte) and a cell possessing abundant mitochondria (m) but no bacteria. Scale bar = 0.5 μm.

observed, while mitochondria are rare. Bacteriocyte nuclei are generally quite small, having diameters of approximately 5 microns.

Also associated with the bacteriocytes are numerous intracellular pigment granules, 6–14 microns in diameter; we have not observed any such granules within the ciliated epithelial cells of the gill. Each bacteriocyte, however, may contain from one to several of these granules (Fig. 3A), which are surrounded by numerous bacterial endosymbionts (Fig. 3B). When thick sections of unstained gill are viewed with light-level microscopy, the pigment granules have a distinct yellow-brown coloration. Staining these sections for occult iron causes the granules to turn green, which is a positive result for the presence of iron.

Enzymatic analyses

Activities of ribulose 1,5-bisphosphate carboxylase (RuBPCase), a CO_2-fixing enzyme diagnostic of the Calvin-Benson cycle, were found in the gill tissue of *L. floridana* at levels comparable to those reported in fresh spinach leaves (Wishnick and Lane, 1971). Catalytic activity increased linearly with increasing volumes of homogenate, indicating enzyme proportionality (Fig. 4). From the substrate saturation curve determined for ribulose 1,5-bisphosphate, the apparent K_m was estimated to be 0.38 m*M* and the V_{max} 3.4 units/g wet weight tissue. This substrate becomes inhibitory at concentrations exceeding approximately 1.2 m*M*, consistent with substrate inhibition values for RuBPCase from other sources (Wishnick and Lane, 1971). Omitting the activity values measured above 1.2 m*M* ribulose, 1,5-bisphosphate, double reciprocal plots yielded quite different values of 4.25 m*M* and 18.3 units/g tissue for the apparent

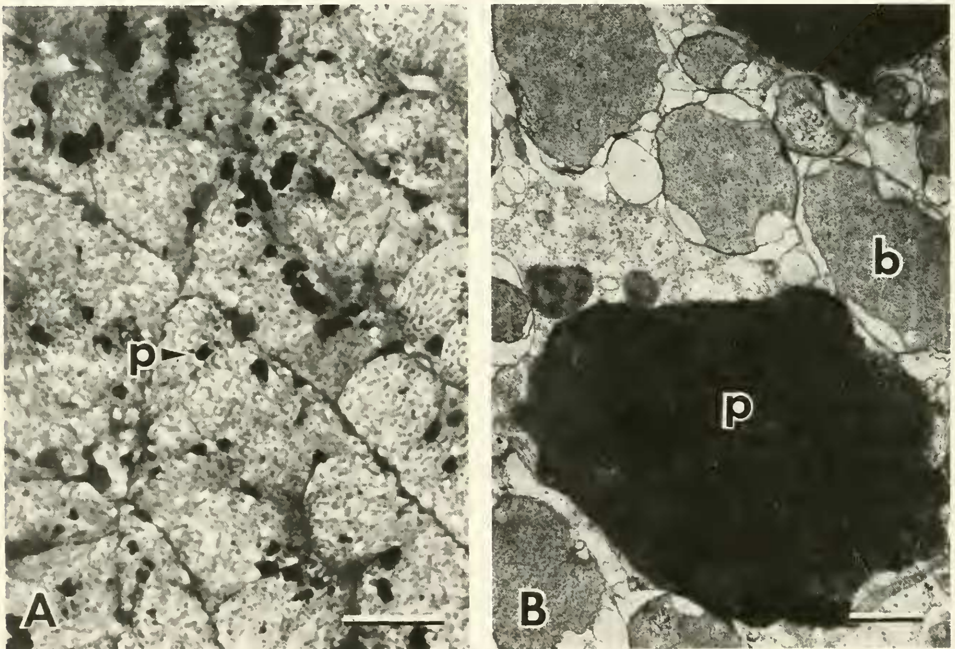

FIGURE 3. A. Light-level micrograph demonstrating the high numbers of pigment granules (p) within the bacteriocytes of the demibranch. Scale bar = 50 μm. B. Higher magnification shows the electron-dense pigment granules in close proximity to the bacteria (b). Scale bar = 1 μm.

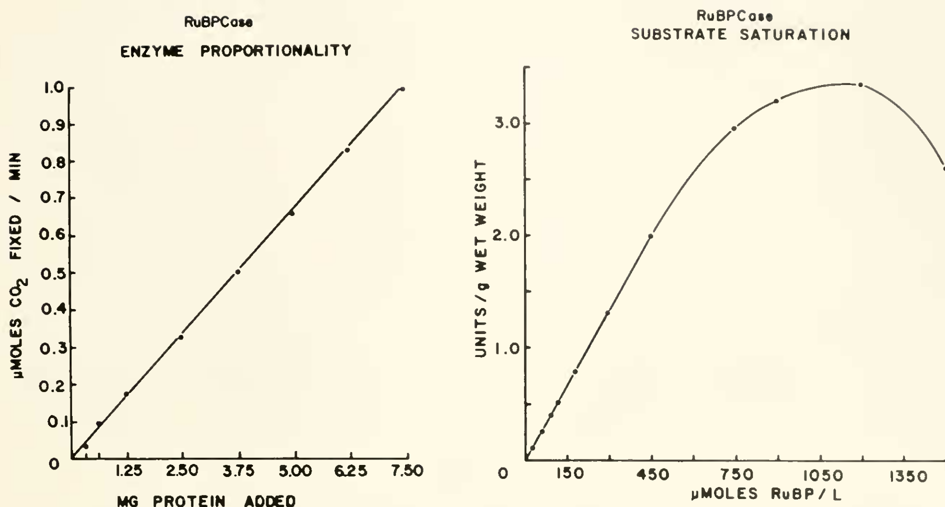

FIGURE 4. Enzyme proportionality and substrate saturation curves of ribulose 1,5-bisphosphate carboxylase (RuBPCase). Ribulose 1,5-bisphosphate becomes inhibitory at concentrations exceeding 1.2 m*M*, resulting in an approximate K_m and V_{max} of 0.38 m*M* and 3.4 units/g, respectively for the uninhibited portion of the curve.

K_m and V_{max}. Phosphoribulokinase, a second enzyme of the Calvin-Benson cycle, catalyzes the phosphorylation of ribulose-5-phosphate to ribulose 1,5-bisphosphate, and activity of this enzyme was also present in gill homogenates (Fig. 6). The activity was proportional to the amount of homogenate added, and a double reciprocal plot yielded a K_m of 0.38 m*M* for ribulose-5-phosphate and a V_{max} of 0.93 units/g wet weight. The presence of these two enzymic activities is consistent with a potential for net CO_2 fixation *in vivo* in the gill tissue.

Results of our enzymatic analyses for APS reductase and ATP sulfurylase are given in Figure 5. APS reductase catalyzes the production of adenosine phosphosulfate from AMP and sulfite, and a double reciprocal plot gave a V_{max} of 2.1 units/g and a K_m value of 0.26 μM for sulfite. ATP sulfurylase phosphorylates adenosine phosphosulfate to form ATP and sulfate; V_{max} and K_m values were 2.9 units/g wet weight gill tissue and 0.13 μM, respectively. Strong enzyme proportionality was observed for both activities. Although the enzyme rhodanese is not as reliable an indicator of sulfur-based energy metabolism as the two enzymes above, it is noteworthy that we were able to qualitatively demonstrate the presence of this enzymic activity in gill homogenates also. However, since the assay technique did not give linear reaction rates we cannot report a quantitative value.

Finally, nitrite reductase, which catalyzes the ferredoxin-dependent formation of ammonia from nitrite, was also measured in *Lucina* gill tissue at a level of 9.4 units/g (V_{max}). The K_m value for nitrite was determined to be 4.0 μM (Fig. 6).

Energy dispersive X-ray analysis

Sulfur was shown to be the dominant inorganic element in air-dried gill tissue (Fig. 7). Attempts to localize the sulfur in specific regions of the tissue by dot mapping suggested that the element was uniformly distributed; using this technique there was no evidence for the existence of concentrated sulfur deposits within the yellowish-

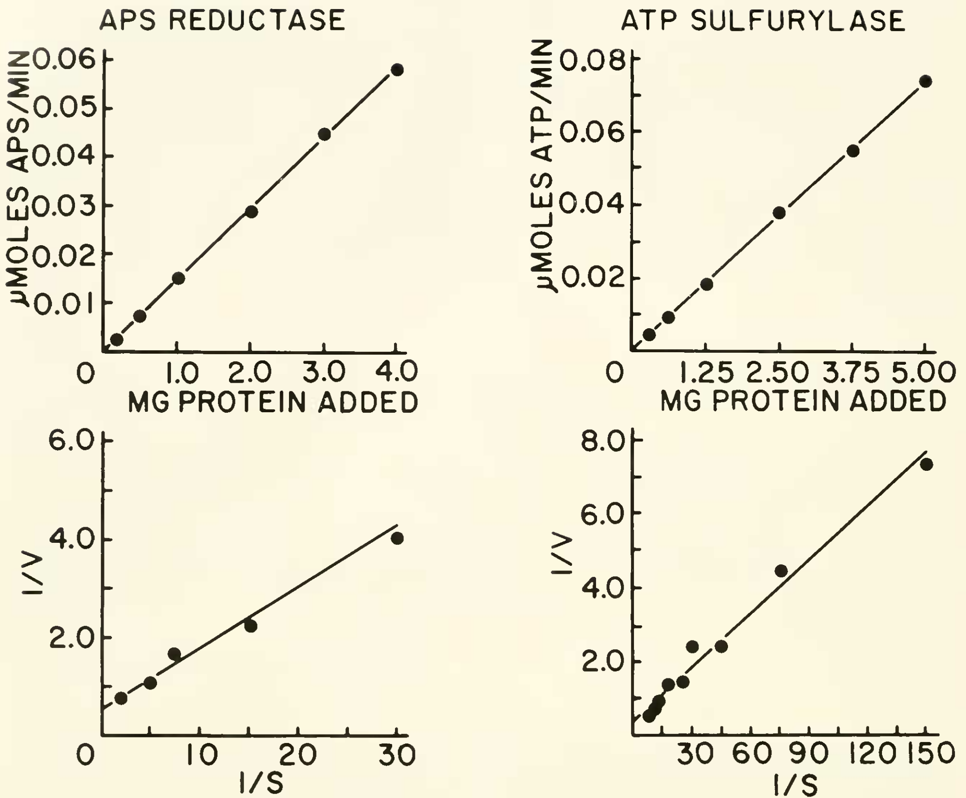

FIGURE 5. Enzyme proportionality and double reciprocal plots of APS reductase (left) and ATP sulfurylase (right). Both enzymes exhibit proportional activities, which increase linearly with the amount of added homogenate. Double reciprocal plots give V_{max} and apparent K_m values of 2.1 units/g and 0.26 μM for APS reductase and 2.9 units/g and 0.13 μM for ATP sulfurylase.

brown pigment granules. Leaching of sulfur from the tissue, as indicated by reduction in size of the sulfur peak, occurred if the samples were processed with an acetone dehydration series and critical point drying. The aluminum peak seen in the elemental spectral scan is an artifact due to the aluminum stub on which specimens were mounted.

Discussion

The above results support the existence of bacterial endosymbionts in the gills of *Lucina floridana.* Confinement of these bacteria to vacuoles within the eucaryotic cells (Fig. 1) is the same morphological arrangement recently reported by Cavanaugh (1983) for gram-negative, symbiotic bacteria in the marine bivalves *Calyptogena magnifica, Solemya velum,* and *Lucinoma annulata* and by Felbeck (1983) for *Solemya reidi.* With the exception of rhodanese (which can also serve as a detoxifying enzyme in certain eucaryotes; Sorbo, 1953), the enzymes that we have demonstrated in the gill tissue of *L. floridana* are normally characteristic of chemoautotrophic sulfur bacteria. RuBPCase and ATP sulfurylase activities have been measured in the trophosome tissue of the vestimentiferan tube worm *Riftia pachyptila* by Felbeck (1981),

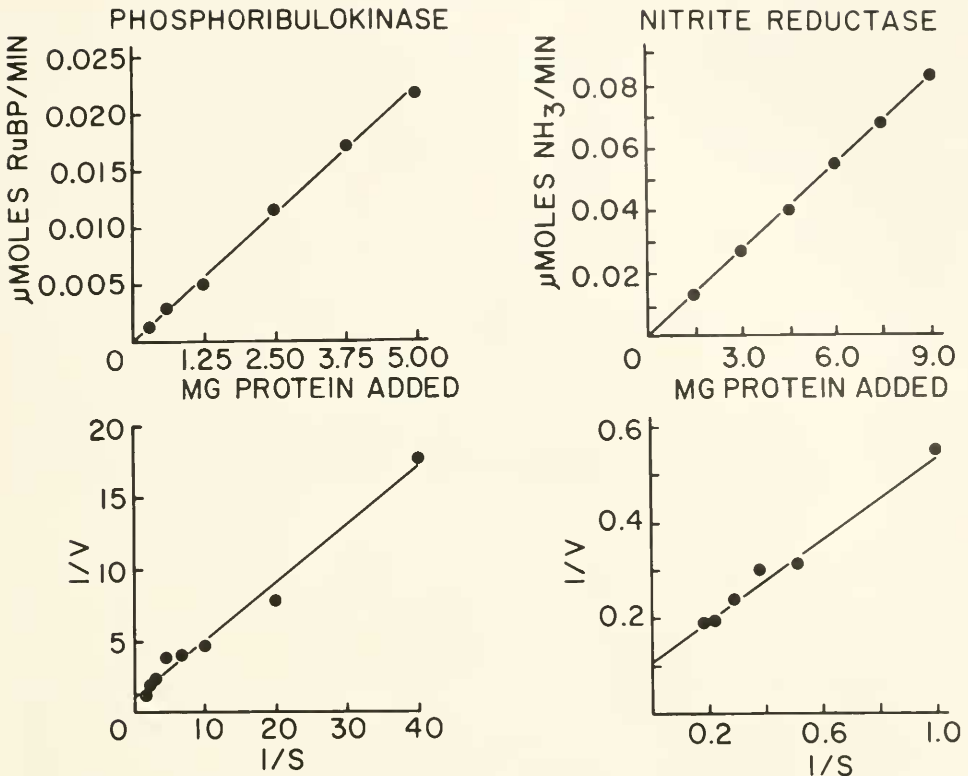

FIGURE 6. Enzyme proportionality and double reciprocal plots of phosphoribulokinase (left) and nitrite reductase (right). The calculated V_{max} and apparent K_m were 0.93 units/g and 0.38 μM for phosphoribulokinase and 9.4 units/g and 4.0 μM for nitrite reductase. Again, excellent enzyme proportionality was observed in both cases.

the pogonophoran *Lamellibrachia barhami* (Felbeck *et al.*, 1981), marine oligochaetes of the genus *Phallodrilus* (Felbeck *et al.*, 1983b), and the bivalves *Calyptogena pacifica, Lucinoma annulata, Parvilucina tenuisculpta, Solemya panamensis,* and *S. reidi* (Felbeck *et al.*, 1981; Felbeck, 1983). For several of these animal-bacterial symbioses, the enzymes phosphoribulokinase and APS reductase also have been documented (for review, see Felbeck *et al.*, 1983a). In the present study with *L. floridana* RuBPCase levels (3.4 units per gram fresh weight of tissue) are the highest reported thus far, with the activities for the other three enzymes falling well within the range of currently-existing values for similar symbioses (Felbeck *et al.*, 1983a). We also report here the presence of nitrite reductase, which has not been noted in any of the other studies above. Thus, it is likely that these bacterial endosymbionts provide *L. floridana* with the potential for chemoautotrophic metabolism fueled by sulfur oxidation. To substantiate this relationship, however, one should be able to demonstrate a sulfide-dependent synthesis of ATP and NADPH in the bacteriocyte, as well as the subsequent release of reduced carbon compounds from the bacteriocytes and transport to other tissues of the host.

For significant sulfide oxidation to occur within the tissue of *L. floridana* the animal must have access to both HS^- and O_2 in its immediate environment. The

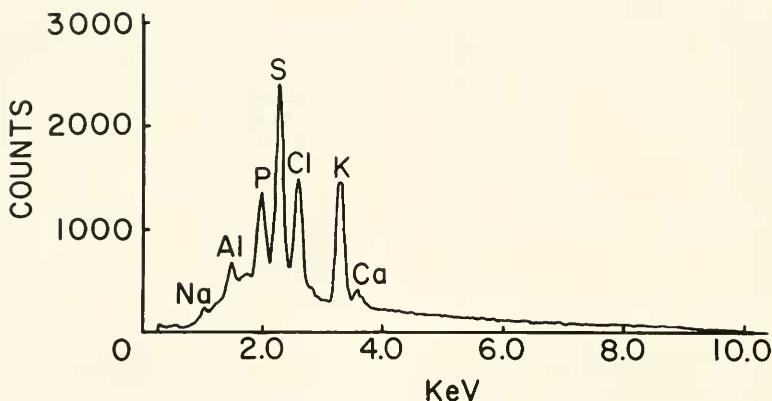

FIGURE 7. Energy dispersive X-ray analysis of air-dried, freeze-fractured *Lucina* gills. Period of analysis is 100 seconds. Sulfur (K_α = 2.3 KeV, K_β = 2.5 KeV) is clearly the dominant inorganic element, while Fe (K_α = 6.4 KeV, K_β = 7.1 KeV) is not detectable. The Al peak is an artifact due to the aluminum stub used for mounting the specimen.

abundance of sulfide is evident from our analyses of core samples taken from the habitat (Table I), and we have observed that the bivalve typically resides in close association with the O_2-releasing root systems of the seagrasses in these sediments. The size of oxygen-containing zones around the roots, in what is otherwise anoxic sediment, is dependent on the magnitude of O_2 flux from the roots and the porosity of the sediment, both of which can be quite variable (Armstrong, 1970). While these oxidized rhizospheres may serve to detoxify potential phytotoxins (*e.g.,* HS^-) as they come into contact with these zones (Armstrong, 1970), it seems reasonable that such rhizospheres could also serve as an O_2 source for *L. floridana;* we have not observed inhalant siphon tubes constructed by the bivalve of sufficient length to allow access to the oxygen in the overlying waters. Thus, while the actual oxygen concentration to which *Lucina* might be exposed has not been measured, it is clear that the animal's location in the sediment could place it at an oxygen/sulfide interface.

As is the case with other lucinids, *L. floridana* appears to have a functional gut. However, Allen (1958) described the digestive system of lucinids as being simplified, with reduction of palps, loss of sorting area, and reduction in the number of restrictions between the stomach and digestive diverticula. Allen suggested that these reductions/alterations of the gut were to facilitate the acceptance of larger food particles like detritus which is common in *Thalassia* beds (Fenchel, 1977); we have indeed noted small amounts of detritus in the gut of freshly-collected specimens. Allen further emphasized that the loss of sorting mechanisms would be expected in an environment where food supply was low and all available particulates must be accepted. Thus, the selective advantage to *L. floridana* of a chemoautotrophic potential in such a habitat would seem clear.

The presence of iron, localized in the pigment granules of *L. floridana* gills using histochemical staining, suggests that respiratory pigments (*e.g.,* myoglobin, hemoglobin) and/or even iron-containing cytochromes could be components of these electron-dense structures. Iron was not detected by energy dispersive X-ray analysis; neither the K_α peak at 6.4 KeV nor the K_β peak at 7.1 KeV was resolvable. However, this observation would not be unexpected if the iron present in these granules was indeed complexed with specific proteins. Under this condition, the iron concentration would be extremely low and possibly below the detection limit of the instrument. Sulfur,

on the other hand, was readily detected by X-ray analysis and was shown to be the dominant inorganic element in air-dried gill tissue. We were unable to specifically localize deposits of this element in the tissue using dot mapping. While structures like the pigment granules should have been within the resolving power of the technique, elemental sulfur deposited within vacuoles of the symbiotic bacteria (as is commonly observed in the Thiobacilli; Trudinger, 1969) would not have been localized due to their small size.

The occurrence of these pigment granules in the gills of lucinid clams has been known for some time. Allen (1958) noted the presence of "brown pigment granules" in the gill tissue of lucinids but offered no possible function. Read (1962) reported that the granules found in the "dark purplish" gills of *Phacoides pectinatus,* another lucinid, contained iron and identified the pigment granules as hemoglobin based on data obtained from absorption spectra and oxygen-combining properties of whole gill homogenates. Jackson (1973) noted the unusual dark coloration (presumably due to the presence of similar pigment granules) of the gills of the lucinids *Parvilucina costata, Lucina pennsylvanica, Anadontia alba, Codakia orbicularis,* and *Ctena orbiculata* and also concluded that the gills contained hemoglobin. Neither author, however, demonstrated clearly that the pigment granules were the source of the hemoglobin. We are currently isolating both bacteriocytes and pigment granules using cell dissociation and density gradient centrifugation in order to approach this question, as well as other ones regarding the proposed sulfide-driven, chemoautotrophic metabolism in this tissue.

One very interesting aspect regarding the morphological placement of the bacteriocytes within the eulamellibranch gill of *L. floridana* is that these cells are restricted to the interior of the organ and are not found in the ciliated epithelium (Fig. 2). The cells composing the epithelium are devoid of bacteria, yet have high densities of mitochondria. Bacteriocytes have a similar locus in the gill of the protobranch bivalve *Solemya* (Cavanaugh, 1983; Felbeck, 1983), such that again the bacterial-containing cells are shielded from the external environment by mitochondria-rich cells (or at least by cytoplasmic extensions of these cells). Since lucinids are known to be stenohaline osmoconformers (Jackson, 1973), an epithelium rich in mitochondria presumably would not be required for active, transepithelial movement of ions. Thus, we propose that one possible reason for such a spatial arrangement is that the gill epithelium may perform an oxygen-scavaging role, protecting oxygen-sensitive enzymes found in the deeper bacteriocytes from high O_2 concentrations. An analogy can be drawn to the leaf morphology of C_4 plants, where the Calvin-Benson cycle is localized in bundle-sheath cells surrounded by an outer layer of mesophyll cells. Since RuBPCase can exhibit both oxygenase and carboxylase activities due to competition between O_2 and CO_2 for the same active site, CO_2 fixation proceeds most efficiently when the intracellular ratio of CO_2 to O_2 is high, allowing normal hexose assimilation (Chollet, 1977). An opposite ratio promotes glycolate formation and the wasteful process of photorespiration. Thus, the high CO_2:O_2 ratio maintained in bundle-sheath cells is functionally beneficial to C_4 plants, and the location of bacteriocytes within *L. floridana* gill tissue could foster efficient functioning of bacterial RuBPCase for similar reasons. Reduced O_2 diffusion to the bacteriocytes might also be beneficial to other bacterial, oxygen-sensitive enzymes like nitrate reductase (Stouthamer, 1976) and sulfur oxidase (Kelly, 1982). Furthermore, if work with isolated pigment granules reveals the presence of oxygen-binding proteins, then these structures could serve as a source of sequestered oxygen to be delivered to bacteriocytes for processes like sulfur oxidation. It should be noted that a high-affinity hemoglobin has recently been documented as an intracellular component of *Solemya velum* gills (Doeller *et al.,* 1983).

Based on average values for population density, grams of fresh gill tissue per individual (0.42 ± 0.07; 95% CI, n = 8), and half-maximal velocities of RuBPCase as determined *in vitro* (1.51 ± 0.26 units/g tissue; 95% CI, n = 8), *L. floridana* could potentially contribute 336 ± 96 g C/m^2/year (95% CI) to the gross carbon fixation of the seagrass beds, a component of gross productivity that has not been considered up to now. Of course, this fixation value is only an estimate, since *in vitro* enzyme activity values are at best only reflective of *in situ* metabolic potential. The contribution to net carbon fixation of seagrass ecosystems, if any, can only be assessed after completion of energy budget studies designed to answer how reduced carbon is partitioned among growth, storage, and routine metabolism in *L. floridana*.

Acknowledgments

We gratefully acknowledge the use of the University of Southwestern Louisiana Electron Microscopy Center and the cooperation of the Florida Department of Natural Resources, Division of Recreation and Parks, during our collections of *L. floridana* specimens. We thank Mr. John Blackson for technical assistance and advice regarding the X-ray analysis data.

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